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Project Summary

Mutagenesis Screening of Pesticides Using *Drosophila*

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Ruby Valencia

Drosophila melanogaster males were exposed by feeding (plus contact and possibly inhalation). The genetic test found most sensitive and appropriate was the sex-linked recessive lethal test. For this, males of the Canton-S wild type stock were exposed. They were mated individually and brooded to sample the entire range of germ cell developmental stages. A very large number of tests (over 7000) was accumulated for each compound in two or more replicate experiments. Concurrent negative controls were done with each, and positive controls were run occasionally. Thirty pesticides and seven other miscellaneous compounds were tested and four reference mutagens were run through the system, some of the latter at a series of "doses" (exposure concentrations). Table 1 lists them all, with the results.

Of the 15 pesticides (listed first in the table) which could be tested at adequate concentrations, four (captan, folpet, bromacil, and simazine) were found to be weak mutagens. One (cacodylic acid) was questionable but called negative. The rest of the pesticides were so toxic that only very low concentrations (0.1-5 ppm) could be used (usually for a reduced exposure time), and those are not considered adequately tested, in view of results obtained with reference mutagens at these concentrations.

Two of the miscellaneous compounds (Tris and $PtCl_4$) were found to be potent mutagens. The rest were negative.

This Project Summary was developed by EPA's Health Effects Research Laboratory, Research Triangle Park, NC, to announce key findings of the research project which is fully documented in a separate report of the same title (see Project Report ordering information at back).

Introduction

The mutagenicity tests carried out in this laboratory using *Drosophila* were part of a larger program involving several test systems in several laboratories. It was not known how *Drosophila* systems would compare with others in terms of sensitivity to detect mutagenicity. Neither was it known which genetic endpoints (in *Drosophila*) would be most adequate in a screening test. It was recognized that pesticides (especially the insecticides) would pose special problems for genetic tests with fruit flies. The *Drosophila* screening system itself was therefore undergoing definition and simplification during the course of these studies.

The original plan was to test each compound in a stepwise manner, as follows:

1. Dominant lethal test (the fastest). Stop if positive.
2. Chromosomal alteration test (a one-generation test). Stop if positive.
3. Sex-linked recessive lethal test.

It became clear, however, that the first two tests were not highly sensitive, and were not easy to do. Thus, the lethal test had to be done in all cases. At the same time, evidence was mounting

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(especially Vogel and Sobels, 1976) that the sex-linked recessive lethal test is by far the most sensitive *Drosophila* test. This type of mutation is frequent, involving some 800 genes on the X chromosome, and varied in nature, including both "point" mutations and chromosomal alterations. We feel that the sex-linked recessive lethal test alone should be an adequate probe for mutagenesis in *Drosophila*.

Many people have been hesitant to accept results with an insect as relative to man. It was thought that insect metabolism was probably too different. These objectives have been considerably reduced by the discovery that *Drosophila* has enzymes which carry out metabolic activation similar to that effected by mammalian enzyme extracts (Vogel and Sobels, 1976).

The toxicity of many of the compounds did cause serious problems, and resulted in a "no test" conclusion for some of the compounds. For those which could be adequately tested, the *Drosophila* results compared quite favorably with the other systems, picking up four weak mutagens and one questionable mutagen. All of these except one were picked up by at least one other system. Two were found positive in all systems.

Table 1. *Summary of Compounds Tested*

Pesticide	*Concentration (ppm) in feeding solution*	*Mutagenesis result*
Bromacil	*2, 3, 5, 2000*	+
Captan	*2, 3, 2000*	+
Folpet	*2, 3, 2000*	+
Simazine	*3, 5, 2000*	+
Cacodylic acid	*3, 500, 100*	-?
Dicamba	*3, 2000*	-
DMSA	*3, 1500*	-
Methoxychlor	*1000*	-
Monuron	*4, 1000, 2000*	-
MSMA	*2, 3, 4, 1500, 2000*	-
Quintozene (PCNB)	*3, 4, 8, 2000*	-
Trifuluralin	*2, 3, 1000*	-
Siduron	*100*	-
Acephate	*10*	-
Carbofuran	*10*	-
Dimethoate	*1, 5, (10)*	-
Methomyl	*4, (10)*	-
Aspon	*5*	-
Azinphos-methyl (Guthion)	*0.25-1.0*	-
Chlorpyrifos (Dursban)	*0.1*	-
Demeton	*1*	-
Dinoseb	*0.5, 1.4*	-
Disulfoton	*1*	-
Ethyl parathion	*0.25, 0.5*	-
Fenthion	*0.1, 0.25*	-
Malathion	*0.25, 0.5*	-
Monocrotophos (Azodrin)	*2, 3*	-
Phorate (Thimet)	*0.5-3*	-
Trichlorfon	*1*	-
Miscellaneous Compounds		
Safrole	*100*	-
1'Hydroxy Safrole	*1000*	-
1'Hydroxy Safrole 2,3 epoxide	*1000*	-
Direct Black 38	*2000*	-
Tris (2,3-dibromopropyl phosphate)	*4, 10, 100, 1000*	+
Benzidine dihydrochloride	*1000*	-
$PtCl_4$	*100, 500*	+
Reference Mutagens		
EMS (Ethyl methanesulfonate	*2, 4, 10, 100, 200, 400*	+
EI (ethylenimine)	*2, 4, 10, 30, 50*	+
TMP (trimethyl phosphate)	*1000*	+
TMP (trimethyl phosphate)	*100, 300*	-
DBE (1,2-dibromoethane)	*5, 10, 50*	-?

Exposure Methods

Initial trials were made of several other exposure methods (aerosol spray, contact, injection), but the method chosen as most appropriate was feeding. Since flies walk on their food, contact is always involved, and when vaporization occurs, the test substance is also inhaled. The method thus seemed more complete and exposure more certain, and it better mimicked human exposure routes.

It was shown several years ago by Lewis and Bacher (1968) that feeding of mutagens in glucose solution is effective. They fed flies in vials with a bit of soaked tissue (such as Chem-Wipes). We chose to feed in small disposable petri plates with a disc of glass fiber filter in the bottom. This permitted easy observation and counting of dead flies for toxicity information.

At first, flies were exposed (toxicity permitting) for 48 hours. Later, to make ingestion more certain, the time was extended to 72 hours. Flies cannot survive for more than 24-36 hours without drinking, but in 72 hours, they should be obliged to ingest more. Contact and inhalation were also thus extended.

Compounds were dissolved, when possible, in 1% aqueous glucose. During the first year, DMSO was used as the initial solvent for compounds not water soluble. A decision was later taken to avoid DMSO if possible. In either case (with or without DMSO) many compounds were used in suspension rather than in solution.

The concentrations were chosen by the following criteria. If toxic at 2000 ppm or below, a concentration giving as near as possible to 50% mortality at 72 hours was chosen. For very toxic compounds, the duration of exposure was reduced as well as the concentration. For non-toxic compounds, 2000 ppm was chosen as a reasonable upper limit.

Sex-linked Recessive Lethal Test

Canton-S wild type males were exposed. "CS" stock was used because it has had a low spontaneous frequency

over many years of use. The frequency averages about 0.15%, with variations rarely exceeding 0.1% and 0.3%.

Treated males were mated to "FM6" females. The X-chromosome of this stock is marked with yellow, white and Bar and carries a complex of inversions.

Heterozygous F_1 females were mated individually to FM6 males (brothers or stock males) and the F_2 of each was observed for the presence or absence of "+" males. Any culture having no or less than 5% of the expected number of males was considered a lethal case and was confirmed by repeating the test with four individual heterozygous F_2 females mated to FM6 males.

Each treated or control male was transferred to new sets of FM6 females at intervals of 4, 3, 3, and 4 days to produce 4 broods of progeny. The different broods sample germ cells in gradually earlier stages of development at the time of exposure. Mature sperm are sampled in Brood 1, spermatids in Brood 2, spermatocytes in Brood 3, and spermatogonia in Brood 4. These are not "clean," but rather "rough" samples, due to the length of the mating times, the fact that chemicals may remain in the body beyond the external exposure time and that some agents affect the rate of spermatogenesis.

"Cluster" Detection and Handling

Treated and control males were numbered, mated, and transferred individually and the F_1 daughters of each were mated as a "family." This is done in order to detect cases where more than one lethal is produced by one male. These cases will be referred to as "multiples" (as opposed to "singles" and "nulls"). A multiple can be due to multiple individual mutations (MIM) or to a single mutation event in a gonial cell, which then reduplicates and produces two or more sperm cells carrying the same lethal (a "cluster"). The distinction (if possible) between these two circumstances is very important when attempting to detect low-level mutagenicity. With potent mutagens, the contribution of clusters is insignificant, and mass matings of treated and control males are the rule. When exposure is simple (as with radiation), and many flies can be exposed, the procedure is often to test only one daughter of each treated or control fly, this avoiding both MIMs and clusters. Exposure is not simple with chemicals, and other precautions must be taken.

The following procedure was applied to this data regardless of the recognized pitfalls. Whenever a multiple was found, a statistical method (devised by Seymour Abrahamson) was applied, which yields the probability (binomial expectation) of that number of mutations arising independently in a single male given (a) the number of males in that particular treated or control group which produced progeny, (b) the average number of F_1 females tested per P_1 male, and (c) the mutation frequency.

If the number of lethals in the multiple greatly exceeded probability, we considered it a cluster and did not count these lethals.

In addition, however, it was often necessary to make some subjective judgments on particular compounds. When the lethals in the replicate treated groups occurred in multiples, while those in the concurrent control group did not, this might have indicated "spotty" exposure.

Number of Chromosomes Tested

The number of tests needed per compound or "compound equivalent," which would be any variant on a compound, such as dose level, germ cell stage, or exposure method, depends mostly upon (a) mutagenicity—the more mutable, the fewer tests needed to prove it—and (b) the increment in frequency desired to detect.

When the control rate is 0.15%, and when it is desired to detect (and prove statistically) an equal induced frequency (a doubling of background), then about 9000 treated and 9000 control chromosomes should be tested.

Dominant Lethals

Methodology was developed for dominant lethal screening and tests were performed on simazine and dicamba, using very low concentrations. Simazine gave possibly positive results. This test, however, was abandoned since it was time-consuming and would probably rarely help avoid future testing.

Chromosome Loss, Rearrangement and Non-disjunction

The test is relatively fast, requiring only one generation, and appears to be simple. In use, however, very large numbers are required and interpretation of results is difficult, due to the several different types of variants and their quite different meanings in terms of cytogenetic events. Since the recessive lethal test also picks up chromosomal rearrangements, it was decided that this separate test was not worth the time and effort required.

Results and Conclusions

The pesticide results fall into three categories:

(a) Those which were tested at concentrations above 10 ppm for at least 24 hours and which yielded results indicating a weak mutagenic effect. These were captan, folpet, bromacil, and simazine, plus possibly, cacodylic acid.

(b) Those which were presumably tested adequately (as above) but which yielded negative results. These were dicamba through carbofuran in Table 1.

(c) Those which may not have been adequately tested, since they were highly toxic, were used at very low concentrations, and gave negative results.

For the four compounds called positive, the conclusion was based upon the experiments with 2000 ppm. Captan, simazine, and bromacil were tested simultaneously in Run No. 37. Folpet was tested in Run No. 38. Both runs had controls with quite low frequencies, but this appeared to be a true low period for the stock. Run 37 was done with a newly prepared stock and the control was the first of a series giving low values. In all four cases, there were several multiples in the treated series, but none in the controls. These multiples were:

Captan—5 males with 2, 2 males with 3, 1 male with 6
Folpet—4 males with 2, 1 male with 3
Bromacil—3 males with 2, 1 male with 3, 1 male with 4, 1 male with 5
Simazine—4 males with 2, 2 males with 3.

When the Kastenbaum and Bowman statistical test (Mut. Res. 9 [1970] 527-549) is applied to the data for these compounds (using the sum of Controls 37 and 38 to provide approximately equivalent numbers of treated and control tests), the result is as follows.

Captan is significant at the .01 level if the multiple of 6 is included. Without the multiple, it is significant at the .05 level.

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Bromacil is significant at the .01 level if the multiples (one of 4 and one of 5) are included. It barely misses significance at .05 without them.

Simazine had no multiples greater than 3 and is significant at the .01 level.

Folpet also had no correction factor, but is significant only at the .05 level. Using the Chi^2 test, which is somewhat less conservative than the Kastenbaum-Bowman test, $P = <.02$. More importantly the folpet data shows a peak of mutagenicity in Broods 2 and 3. Using these broods only, the result is highly significant ($Chi^2 = 15$, $P = <.01$).

In all these cases, the deduction of multiples of 4, 5, and 6 is questionable, since there are also several multiples of 2 and 3, while the controls have almost none.

It is possible that exposure may have been "spotty"—i.e., some males may have ingested, contacted, or inhaled more than others, in which case the compounds may be more mutagenic than the averaged results indicate.

Cacodylic acid caused marked sterility of treated males, especially in Brood 3, indicating a cytotoxic effect on premeiotic cells. Unfortunately, it is not possible to know from these experiments whether or not the damage is genetic. The few remaining germ cells showed a reduced mutation frequency. The recessive lethal frequency obtained in the one adequate experiment (Run 52) was actually 3 times the concurrent control value. The latter, however, was exceptionally low, and in this case it was not part of a consistently low control period. (Controls were, in fact, somewhat erratic during that time.) There were no multiples in Control 52 and only one multiple of 3 in the treated males. The result was, thus, considered negative but questionable.

In the tests of miscellaneous compounds, Tris and $PtCl_4$ were clearly mutagenic, with peak effects in Brood 2, indicating an effect primarily on spermatids. All others were clearly negative.

The reference mutagens EMS, EI, and TMP were positive when tested at adequate concentrations. EMS, a very potent mutagen, surprisingly gave frequencies very near control level when tested at very low concentrations (2 and 4 ppm). TMP was negative at 100 ppm and 300 ppm. DBE was tested only at low concentrations and was negative or questionable. It is probable that our exposure technique was inappropriate for this volatile compound. In all these cases, however, there were multiples, indicating possible "spotty" exposure.

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***Michael D. Waters** is the EPA Project Officer (see below).*

The complete report, entitled "Mutagenesis Screening of Pesticides Using Drosophila," *(Order No. PB 81-160 848; Cost: $9.50, subject to change) will be available only from:*

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